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Stapp, C. & G. Spicher 1954. Untersuchungen über die Wirkung von 2,4-D
im Boden [Research on the activity of 2,4-D in the soil].
Zentralblatt für Bakteriologie, Parasitenkunde, &c. II. Abt. Bd. 108.
Heft 4/7, pages 113-126.

Arne K. Lin
Resumé

The stability of the selective hormonal herbicide, 2,4-D, in the soil is certainly one of its most important properties; for only so long as the 2,4-D acid maintains itself in the soil can the desired weed control be effected. The use of 2,4-D is advisable, since it affords a weed-free environment for planting, but it can be used repeatedly only with the assumption that, after the desired period of control, the herbicide is inactivated or removed from the soil. Since the 2,4-D must ^{be} below a certain concentration before planting, this problem deserves a lot of attention. A slight over-abundance in the substrate can ruin the crop (Audus, 1949; Bouillene-Walrand, 1952; Jensen & Petersen, 1952).

It is well known that most 2,4-D disappears from treated soil within a few weeks of treatment. This disappearance is attributed to microorganisms which metabolize 2,4-D. Using the "perfusion" technique, Audus (1949) has plotted the 2,4-D concentration against time; after treatment, he distinguishes three phases: (a) a slight reduction of the detectable 2,4-D, the result of adsorption onto soil colloids; (b) a phase during which the 2,4-D level remains about the same; (c) a rapid disappearance of 2,4-D. The curve is typica

of those reflecting biological processes in the soil [page 1147]

and Audus attributes the "detoxication" to the work of microorganisms.

With repeated treatment of the soil, the second phase is hastened.

Autoclaving or addition of a bactericide inhibits the activity.

In impure culture of soils inoculated with the active organism 2,4-D was broken down in the same way (Newman & Thomas, 1950); Audus (1950:1951) finally isolated an organism which was able to survive on synthetic media with 2,4-D as its only carbon-source. According to his work, this bacterium is obviously a member of the Bacterium globiforme group of Lochead & Taylor. Later Jensen & Petersen (1952) identified two more 2,4-D bacteria, a "Flavobacterium aquatile," and a so-called "Bacterium 2," which they stated bore some relationship to the form described by Audus.

Identification of a 2,4-D Bacterium

Proceeding from the earlier work (Stapp & Freter, 1952; Stapp & Wetter, 1953) also succeeded in isolating the active organism which in pure culture on solid or liquid media was able to inactivate the sodium salt of 2,4-D (when not otherwise specified, "2,4-D" will here signify the sodium salt thereof). Composition of the medium: $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.2%, NH_4NO_3 0.1%, KCl 0.02%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%, 2,4-D 0.1%, Agar 2.0 to 0.1%.

The demonstration of the possibility of fluid-media culture was a significant advance. This was possible by spreading the medium with sufficient surface-area for a good oxygen supply (as the work of Akamine, 1951 and of Stapp & Wetter 1953 indicate is required), and above all an addition of 0.1% agar, which Audus (1949, 1951) had

had already indicated. It is also a good idea to permit the autoclaved culture flasks to stand at room temperature for a day before inoculation so that the media can absorb oxygen.

The yield of the impure culture of 2,4-D bacteria can be enhanced by repeated moistening of the sample with a 0.1% solution of 2,4-D, with a cress test following each moistening) This procedure can be repeated until finally when a "small amount" of the "enriched Soil" is introduced into the flask containing the 2,4-D-containing medium, there is an immediate reduction in the 2,4-D concentration in the flask.

Pure culture at first was difficult, until it became evident that the 2,4-D bacterium is very sensitive to temperature. The unusually low upper limit of temperature tolerance was demonstrated thusly: For each temperature series, 2 cc of sterile medium was inoculated with a young culture, and this was allowed to remain 10 minutes in a constant-temperature water bath. The following different temperatures were used: 25, 30, 35, 40, 45, 50, and 55° C. Solid 2,4-D medium was then inoculated from the temperature-treated aliquot, and the survival of the bacterium noted. The test was replicated four times. In all cases the bacteria survived temperatures of 40° C or less. A ten-minute exposure at 40° C greatly reduced the bacteria, while at 45° C the tolerance limit was already exceeded.

Even in the most diverse soils, the addition of 2,4-D led to a similar increase in the 2,4-D bacteria. This was accompanied, in all impure cultures, by an increase in Bacillus megaterium.

The tables of the "Manual of Determinative Bacteriology" by Bergey, and the so-called "Skerman-key" (1949) or its German translation

by Meyn (1952) were used in the determination of genus to which the isolated 2,4-D bacterium belongs. Both indicate the genus Flavobacterium. Specific determination was considerably more difficult. The key of the "Manual of Determinative Bacteriology" takes us to three different species: F. aquatile, F. breve, and F. solare.

All three are non-motile, do not dilute gelatine, or do so only very slowly, leave litmus unchanged, and do not reduce nitrate to nitrite. Only in their temperature optima are they clearly differentiable.

From a further investigation of properties, it was inferred that our 2,4-D bacterium is closest to Flavobacterium aquatile, insofar as the inference could be drawn on the basis of the published descriptions of properties. We contrast the two species below:

The colony of F. aquatile has a yellow-brown center and a colorless margin; our bacterium has a pure yellow colony.

F. aquatile dilutes gelatine only very slowly, our bacterium not at all.

F. aquatile occurs as singles, pairs, and chains; our bacterium occurs as singles or pairs, not as chains.

F. aquatile tolerates but does not require oxygen; our bacterium requires oxygen.

Above all, they differ in size (or length-width ratio): when F. aquatile is 0.5 micra wide it is about 2.5 long; when our bacterium is that wide, it is only about 0.8 micra long.

The 2,4-D bacterium identified by Jensen & Petersen (1952) as F. aquatile fits the description of the "Manual of Determinative Bacteriology" in size (larger than ours). Curiously, the bacterium of

Jensen & Petersen does not grow on a semisolid agar-medium nor on earth-extract, but develops well on a glucose medium. Our bacterium, on the other hand, does very well on nutrient solution plus 0.1% agar, and is markedly held back when 1% glucose is added.

In an attempt to determine how our 2,4-D bacterium compares with strains which have been determined by other authorities as Flavobacterium aquatile, in a series of tests four of these strains were compared with our bacterium (Table 3). All five strains are very similar, but display a few distinctions, these having to do with chain-formation, colony-color, ability to thin gelatine, and growth in nutrient bouillon.

Strain 1 is closely related to strain 2, and strain 3 to strain 35. Our 2,4-D bacterium is intermediate, with a special form of colony in agar and in gelatine.

In an examination of properties not specified in the "Manual", more extensive differences became evident, when neither acid nor gas formed in the bouillon-carbohydrate media (Table 4). Here (in Table 4) our bacterium is again intermediate, but closer to strain 3 and 35. The decrease or retardation of its development in the presence of glucose and lactose is especially remarkable. Only our 2,4-D bacterium persists on the 2,4-D medium; even after a week's incubation, none of the others develops an adaptation to 2,4-D.

Audus (1949) hypothesized that the long first phase in the breakdown of 2,4-D in previously untreated soil was the time during which the normally inactive bacteria adapt themselves to 2,4-D metabolism.

Jensen & Petersen (1952) considered it unlikely that an organism with an enzyme system capable of this break-down would be common in a previously

untreated soil. They considered the 2,4-D bacterium to be a *Flavobacterium aquatile* adapted for the break-down of cyclic compounds.

Since, however, none of the investigated strains of *Flavobacterium aquatile* from untreated soils has ever succeeded in utilizing 2,4-D, we consider it appropriate to look upon our 2,4-D bacterium as new, and propose for it the name *Flavobacterium peregrinum*, new species.

The Break-down of 2,4-D by *Flavobacterium peregrinum*.

It seemed to us very important to compare the activity of pure and impure culture of *Flavobacterium peregrinum*, to see whether the impure culture may eventually contain still other organisms capable of 2,4-D break-down. The possibility arises, that if the 2,4-D bacterium can be induced to break down 2,4-D in fluid medium, the portion of the break-down attributable to the 2,4-D bacterium (Strain D) can easily be ascertained.

The nutrient described on page 114 was doubled in concentration, but with the same amount of earth-extract, and 20 cc placed in each of a number of 100-cc flasks, and autoclaved. One group was inoculated with Strain D, the other with a small amount of a soil sample ~~xxxxxx~~ enriched with the 2,4-D bacterium.

In pure culture the principal phase of 2,4-D break-down usually starts the first day, and after 2.5 days the bacteria have inactivated half of the 2,4-D supplied. In impure culture, on the other hand, the principal phase begins on the third day, and the half-way point is reached between the fourth and fifth days. Therefore *Flavobacterium peregrinum* is solely responsible for the nutrient-break-down, and in this respect *Bacterium megaterium*, which is usually found with it, is insignificant.

It is widely known that the disappearance of 2,4-D in various soils proceeds at different rates. However, the results of research on the have reason for these differences, up until now, ~~had~~ been equivocal.

There follows a short review of literature: Kries, 1947; Akamine, 1951; Stapp & Wetter, 1953; Mitchell & Marth, 1946; Hanks, 1947; Brown & Mitchell, 1948; Krone & Hammer, 1947.

With the aid of fluid culture it can be shown that soils of various origins contain a factor which, depending on the origin of the soil, differentially affects the rate of growth of the 2,4-D bacterium. samples

The finely sifted soils were placed in twice their weight of distilled water, were heated 30 minutes in the autoclave, centrifuged, and filtered through a membrane filter (No. 10 of the membrane filter company in Gottingen). A part of the resulting extract was added to a similar amount of the doubly-concentrated nutrient solution, and 20 cc placed in a number of 100-cc. flasks, sterilized, inoculated with Flavobacterium peregrinum, and incubated at 25°C.

The addition of soil extract speeds up the breakdown (see fig. 3a. and 3b.); if the time is propitious, the time required to break down half the 2,4-D is reduced from 72 to 20 hours. The most strongly active soils were, in our experience, humic to very humic fine sandy loams, with no carbonates, probably alluvial, and used as meadows. The results of several other workers have been taken to indicate that the rate of 2,4-D inactivation is dependent on the sum of several factors whose total effect determines the effective activity. In our experience, one of these factors, as already pointed out by Audus (1952) is a water-soluble growth-stimulant which occurs in varying amounts. Our factor

Table 2. Comparison of the *Flavobacterium aquatile*, *F. breve*, *F. solare*, and *F. peregrinum*.

Table 3. Comparison of the characteristics of four strains of *Flavobacterium aquatile* and *Flavobacterium peregrinum*.

	Strain 1	Strain 2	Strain 3	Strain 35	Fl. peregrinum
<u>Bacillus</u>	-	-	-	-	-
of <i>F. aquatile</i>	-	-	-	-	-
<u>Bacillus</u>	-	-	-	-	-
0.5x0.8-1.8 micra	0.5x0.8-1.8 micra	0.5x0.8-2.4 micra	0.5x0.8-2.4 micra	0.5x0.8-2.4 micra	0.5x0.8-2.4 micra
non-motile	non-motile	non-motile	non-motile	non-motile	non-motile
Gram-negative	Gram-negative	Gram-negative	Gram-negative	Gram-negative	Gram-negative
single, pairs, and chains up to 8 micra long	singles, pairs, and chains up to 12 micra long	singles, pairs, and chains	singles, pairs, and chains	singles, pairs, and chains	singles, pairs, not chains
Gelatine plate	brownish, smooth round, lustrous	brownish, smooth round, lustrous	brownish, smooth round, lustrous	brownish, smooth round, lustrous	brownish, smooth round, lustrous
Gelatine-stab	brownish surface-growth no liquefaction	brownish surface-growth no liquefaction	brownish surface-growth slow liquefaction	brownish surface-growth slow liquefaction	brownish surface-growth no liquefaction
Nutrient agar	brownish smooth lustrous, not slimy	brownish smooth lustrous, not slimy	light yellow, smooth lustrous, not slimy	light yellow, smooth lustrous, not slimy	light yellow, smooth lustrous, not slimy
Nutrient bouillon uncharged	slightly turbid slight yellow precipitate	slightly turbid slight yellow precipitate	turbid white precipitate	turbid white precipitate	turbid white precipitate
Litmus	unchanged	unchanged	unchanged	unchanged	unchanged
Potato	poor growth not formed	poor growth not formed	yellow streaks	yellow streaks	yellow streaks
Indole	not formed	not formed	not formed	not formed	not formed
Nitrate-reduction	not nitrite	no nitrite	no nitrite	no nitrite	no nitrite
Oxygen-requirement	positive requirement	positive requirement	positive requirement	positive requirement	positive requirement

Table 4. Comparison of the characteristics of four strains of *Flavobacterium aquatile* and *Flavobacterium peregrinum*.

	Flavobacterium aquatile			Flavobacterium peregrinum
	Strain 1	Strain 2	Strain 3	Strain 35
Nitrate agar	brown-yellow (surface only)	brown-yellow poor growth (surface only)	light yellow good growth (surface only)	pure yellow slow growth (surface only)
Dextrose agar	brown yellow poor growth (surface only)	brown yellow poor growth (surface only)	light yellow good growth (surface only)	pure yellow slow growth (surface only)
Bouillon	unchanged	slightly turbid white precipitate	slightly turbid white precipitate	turbid slight white precipitate
Bouillon plus soluble starch	unchanged	unchanged	unchanged	unchanged
Bouillon plus glucose	turbid	turbid	turbid	turbid
Bouillon plus saccharose	turbid	slight yellow precipitate	much white precipitate	much white precipitate
Bouillon plus lactose	turbid	slight yellow precipitate	much white precipitate	much white precipitate
Bouillon plus glycerol	unchanged	unchanged	unchanged	unchanged
Bouillon plus manitol	unchanged	unchanged	unchanged	unchanged
Bouillon plus potassium nitrate	slightly turbid	slightly turbid	slight growth	slight growth
Peptone water	turbid	turbid	slow growth	slow growth
Mineralized glucose	no growth	no growth	no growth	no growth
2,4-D nutrient	no growth	no growth	no growth	no growth